Method for Rapid Detection of Microorganisms

By Changing the Shape of Micro-Colonies

BACKGROUND OF THE INVENTION

Field of the Invention

Growing microorganisms in order to detect, enumerate and identify viable cells – bacteria, fungi, actinomycetes – is one of the most widely used procedures in microbiology. The ability to form a colony on or in an appropriate nutrient media is recognized as the most reliable criteria for cell viability. This requires different biochemical reactions and processes like respiration, transportation, synthesis and decomposing of different proteins, carbohydrates, lipids, enzymes, nucleic acids and many other substances, and creation of inner structures in order to finally reach cell reproduction and creation of a colony. The detection of live cells cannot be fully substituted by the simple detection of enzymes (enzymatic methods of detection), purified DNA or RNA (PCR methods), antigen – antibody interactions (Enzyme Immunological Analysis, ELISA, Immunoprecipitation, Immunofluorescence, Phagodiagnostics and others), fatty acids analysis (Chromatography), FTIR spectroscopy or other methods. This is due to the presence of specific molecules or even some working systems of the cell, which can be found in dead cells, cells under deadly stress, or cells without access to some required substances that are therefore restricted in growth potential.

The growth occurs either on artificial or natural nutrient media in solid or liquid form. There is a multitude of different media with selective criteria required for growth and total count of groups or species of microorganisms. They include media for growth, detection and enumeration of total number of bacteria (Tryptic Soy Agar and other), fungi, molds (Sabouraud Dextrose Agar, Potato Dextrose Agar and other), selective media for group of microorganisms like Gram-negative bacteria (MacConkey Agar, Levine EMB Agar and other), Lactobacillius (Lactobacillii MRS Agar) or Salmonella (SS Agar), certain microbes like E. coli O:157 (Sorbitol MacConkey Agar), Vibrio chelerae (TCBS Agar), Campylobacter (Triple Sugar Iron Agar) and many others. Growth of microorganisms takes from hours to several days, or even weeks to form visible colonies or a visible suspension of cells. Growing periods on nutrient media is the most time consuming process in modern microbiological diagnostics. This significantly decreasing the time of microbiological analysis and saving the high level of reliability (i.e., detection by growth – forming of colonies) is very important for modern medical diagnostics for early diagnostics of humans or animals infections, epidemiology, and detection of antibiotic resistant microbes and so on. Rapid analysis is also needed in food, pharmaceutical, and biotechnological industries for control and prevention of contamination in food, drugs, and medical devices and for environmental monitoring.

This invention introduces a new method of rapid detection of cellular microorganisms during their preliminary short growth on or in nutrient media. The term "colony" or "micro colony" in microbiology means a group of cells appearing from one single cell and consisting only from descendants of that cell. A colony or micro colony can have different shapes: semi-sphere if grown on the surface of solid agar, oval, cone or "star" if grown in solid or semi-solid agar, a dense or diffuse cloud if grown on semi-liquid or liquid media, or flat fibers growing from one center (fungi, actinomycetes, or some bacterial micro colonies). This invention is based on changing the natural shape of micro colonies to a long cylindrical shape by growing the colony in a long and

extremely small micro-channel that enhances the optical characteristics of the micro-colony and strongly improves the micro-colony's visibility (detectability). An array of micro-channels on a glass plate is part of the Sample – Detection Unit (SDU) – a device for trapping microorganisms, allowing growth, and performing analysis. The improvement of the visibility (detectability) of the micro-colonies shortens the time between inoculation and detection of the colony.

Description of the Related Art

There are several different methods and instruments employed to enhance colony visibility. The addition of special, non-toxic substances (artificial chromogenic or fluorogenic substrates) to solid nutrient media changes the color of the micro-colonies or makes them fluorescent, improving their visibility in early growth stages. Some microorganisms like E. coli O:157, Staphilococcus aureus, and Salmonela grows on "Chromagars," a solid or semi-solid nutrient media that specifically changes the color of investigated microorganisms because of artificial substrates for unique enzymes added to media. These substrates are non toxic to the cells and allow normal growth. Toxic artificial substrates such as Tetrazolium salts, Fluorescein diacetate and other substances can be used in later growth stages to colorize cells and make them more clearly visible.

Detection and enumeration of the colonies are done visually or with magnifying devices. Visual detection and enumeration using magnifying devices requires relatively big colonies; from hundreds of microns to millimeters in diameter. Microscopy helps to find micro-colonies that are smaller in size, however, these colonies must be at least tens of microns in size, contain at least several hundred cells, and require at least 5-10 hours of incubation to form.

Detection of microorganisms could also be achieved by dividing a sample into many discrete zones, adding liquid nutrient media, incubating for several hours to several days, adding

indicator substance or counting turbid zones, and calculating concentration (US Patent No. 5,716,798). This method gives a reduction of growth of only 20-40% because it uses relatively large volumes of discrete zones, consisting of large wells on the side of a special flask for growth. A 90% of zone volume related to nutrient media and therefore analyzing sample could be only several milliliters. Therefore, this method is used only for the detection of microbes in human blood because blood samples are very small and their contamination is in a range from single cells to several hundred cells per milliliter. This current invention utilizes thin, long micro-channels that are open on both ends. A large volume of sample is filtrated through a micro-channel plate. The extremely small volume of the micro-channels reduces the required growth time to 1-5 hours, an 80-95% time savings. Growth of microorganisms within these long, thin micro-channels changes the optical characteristics of micro-colonies much faster than growth of micro-colonies in a large flat volume.

Thus, modern microbiology utilizes a few approaches to shorten the required time for microorganism growth and to improve the visibility of colonies; first - employ optimal growth nutrient media, second - add chemical matter in the nutrient media, on a colony, or into the suspension of cells to change the optical characteristics of colonies, and third - employ optical instruments or devices.

There are no methods utilizing the shape of the colony during its growth in order to enhance its optical density (light absorbance). Changing a colony's shape from a regular semi-sphere with a large volume and a large amount of cells to a thin cylinder shape with a small volume and a small amount of cells will strongly reduce the time between inoculation and colony counting. Smaller amounts of cells need a shorter time for their production. The usage of chemicals producing color or fluorescence and optical instruments together with detection of cylindrical colonies would improve visibility and reduce the time required for analysis.

Reducing the time between the inoculation and detection of microorganisms is very important for swift decisions in quality and process control in a multitude of industries including food, biotechnology, medical, microbiology, environmental, biodefense, and scientific research.

SUMMARY OF THE INVENTION

The invention is based on growing micro-colonies of microorganisms in thin, long, micro-channels that are open on both ends, instead of growing colonies on the standard flat surface of solid nutrient media, filter placed on nutrient media, or growth in a relatively large volume of liquid nutrient media.

The shape of a regular micro-colony is usually semi-sphere. The thickness (height) of micro-colonies is crucial to visibility using microscopy as a thick (high) colony has a greater light absorbance, which is the most important optical characteristic of visibility. A long and thin micro-colony has the same light absorbance as a regular semi-spherical colony of the same height – h (see Fig.1: 1 – regular micro-colony; 2 – micro-colony in micro-channel; 3 – filter; 4 – nutrient media). The volume and amount of cells in a long, thin micro-colony is much less, and therefore the time of incubation to create a well visible colony is much shorter than when growing on a flat surface.

The growth of a cylindrical micro-colony can be made on a grid of channels. The diameter of each of these channels needs to be very small, only 4-20 times larger than the size of the investigated cells. A good example of a grid for this purpose is the MCGP – Micro-Channel Glass Plate. The MCGP contains thousands of extremely small, precisely manufactured, long channels. A regular MCGP has 700,000 channels per cm^2. Each channel has a diameter of 10 microns, and a

length 500 microns. In general, the MCGP is attached to a filter (Fig 2) and a sample is filtrated through both the MCGP and filter. Some micro-channels obtain live cells.

After the filtration is completed, the MCGP and filter is placed on a nutrient agar or agar block of solid nutrient media attached to this filter (Fig.5). Nutrient substances from the media penetrate the filter, and fill all micro-channels. Growth of micro colonies starts after this penetration (Fig.2).

Calculations below show the obvious advantage in reducing the time of growth in micro-channels compared with flat surfaces.

The regular shape of colonies growing on flat surfaces of solid nutrient media is, usually, near to semi-sphere. The volume of semi-sphere is $Vss=\P\cdot h^{2}\cdot(R-h/3)$, where Vss-volume of semi-sphere, R-radius of sphere and h - part of radius - height of semi-sphere.

The volume of cylinder (cylindrical colony) is $Vcc=\P\cdot R^{2}\cdot h$, where R-radius of cylinder, h-height of cylinder.

Micro-colony with height (h) 10μk and R=20μk has volume:

$$Vss = 3.14\cdot 10^{2}\cdot(20-10/3) = 5234\mu k^{3}$$

Cylindrical colony with the same height (h=10μk) and R=2.5μk has volume:

$$Vcc = 3.14\cdot 2.5^{2}\cdot 10 = 196\mu k^{3}$$

Thus, the volume of a cylindrical colony is smaller than the volume of semi-spherical micro-colony with the same height by 27 times, yet both have the same light absorbance.

The volume of one cell of Escherichia coli (E. coli) is near to $1\mu m^{3}$. The speed of multiplying of E. coli is around 20 min at optimal temperature, on optimal media. One cell of E. coli can produce 8 cells in one hour, 64 in two hours, 512 in 3 hours, 4096 in 4 hours and 32768 in 5 hours. Thus, one visible micro-colony on a flat surface, containing 5234 cells, can be formed in 4.2 hours. The cylindrical colony with the same height and light absorbance (196 cells) can be formed in 2.5

hours. Therefore, the growth of micro-colonies with a cylindrical shape has a significant advantage as visualization of colonies can be done at much earlier stages.

The visualization of microorganisms in the micro-channels is much faster than in regular tubes or wells of an immunological plate, or other known laboratory devices for microorganism growth, because of the very small volume of micro-channels and the long cylindrical shape. Thus, one cell in a cylindrical micro-channel, with a length 500µk and diameter 10µk ($V=40{,}000\mu k^3$), corresponds to a concentration of 25 million cells per ml ($V=10^{12}\mu k^3$). Forty cells in a micro-channel correspond to the concentration 10^9 cells per ml, which is a well-visible concentration. One cell of E. coli can reach this concentration (40 cells per micro-channel = 10^9 cells per ml) in 1.7 hours.

Experiments show that 10 layers of colorless small cells (for example E. coli) are enough to find visual differences between micro-channels containing cells and empty micro-channels using a regular light microscope, even with a small magnification of X100. A smaller diameter of micro-channel requires a smaller amount of cells to create 10 layers of cells in the channel.

Table 1 represents the number of layers of E. coli that can be produced in micro-channels of different diameters between one and five hours.

TABLE 1

Correlation between time of forming layers of cells in micro-channels and diameter of micro-channel (E. coli, growth at 37°C on TSA; the time of multiplication = 20 min)

Hours of Incubation / Diameter of Micro channel	1 hour	2 hours	3 hours	4 hours	5 hours
2 μk	3 layers	21	171	1365	10920
3 μk	1	9	73	585	4680
4 μk	0.6	5	39	315	2520
5 μk	0.4	3	26	205	1640
7 μk	0.2	2	13	108	860
10 μk	0.1	1	6	50	410

Table 1 shows that 10 layers of cells will be reached in a micro-channel with a diameter of 2 μk in 1.5 hours; in a 3μk micro-channel in 2 hours; in a 4μk micro-channel in 2.3 hours; in a 5μk micro-channel in 2.7 hours; in a 7μk micro-channel in 2.9 hours and in a 10μk micro-channel in 3.5 hours. Thus, the detection and enumeration of long cylindrical micro-colonies, according this invention, can be done 10 – 20 times faster than regular growth, detection and enumeration of colonies.

A micro- colony can start formation regardless of initial trapping position within a micro-channel. Fig. 3 shows four typical positions of a cell in a channel. 1 – Cell was trapped on the surface of the filter and colony formed from the bottom of micro-channel. This formation appears

when nutrient substances just moistens filter. 2 – Cell was trapped on the wall of micro-channel by the force of adhesion or by antibody attached to the wall preliminary to filtration.
The formation of a micro-colony starts only if the micro-channel is completely filled by nutrient substances from wet agar media, or a thick paper filter filled by a nutrient broth. 3 – Cell was trapped on the filter, but was later raised up by liquid media and started forming micro-colony. In all of these cases, one cell formed a micro-colony of cells that were descendants of the first cell in the solid, semi-solid or liquid culture.

The micro-channels containing colonies appear as dark dots (FIG. 6/1) when a regular light microscope is used. The addition of artificial chromo- or fluorogenic substrates to micro-colonies can reduce the time between inoculation and detection as they make micro-colonies much more visible at an earlier stage. Fig. 6 shows the differences between natural non-colored micro-colonies (1), micro-colonies colored by chromogenic substrates or absorbent dyes (Fig. 6/2), and micro-colonies colored by fluorogenic substrate or fluorescent dye (Fig. 6/3). The coloration of micro-colonies inside micro-channels is done by attaching agar or filter paper treated with required substances to the opposite side of the filter attached to the micro-channel plate (Fig. 6). A light microscope sends light through the MCGP(colorless), filter(colorless), and agar(light transparent) (Fig. 6/1 and 6/2), revealing long cylindrical shaped micro-colonies because of the natural light absorption of cells, or due to cells colored by chromogenic substrates or absorbent dyes. A fluorescent microscope (Fig.6/3) sends a shorter wave light (ultra violet, blue or other depending on dye) and accepts long waves of fluorescence (blue, green or red). Therefore, micro-channels with micro-colonies will appear as bright dots on a dark background. The structure of the SDU for fluorescent version is: MCGP (black non fluorescent), filter (black non-fluorescent), and agar (filled by fluorescent indicator). Fluorescence is considered a much more sensitive type of

analysis. Thus, the micro-colonies can be much smaller/shorter than those analyzed with the use of light absorbance.

This invention differs from other methods of detection of colonies by using a glass plate containing hundreds of thousands of extremely small and long micro-channels that are open from both ends (micro-channel plate). The combination of a micro-channel plate and a filter allows cells to be trapped on the surface of the filter, thus allowing colonies to grow inside the micro-channels. Colonies grown inside the micro-channels will obtain a tall cylindrical shape, increasing light absorbency with a smaller volume and cell number than if grown on a flat surface, drastically reducing the amount of time required for analysis. This method is realized with a simple device consisting of a micro-channel plate, a filter to trap cells by filtration from air or liquid, and a frame consisting of several parts (Fig. 4).

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 demonstrates the geometrical differences between a regular semi-spherical micro-colony (left) and a typical micro-colony growing in a micro-channel of a MCGP. The height of both objects is equal (h) but the volume, and consequently the number of cells and the growth time required to reach equal height, is significantly different.

Fig.2 shows three main components of the sampling-detection device: MCGP, filter and agar block (left), and several enlarged micro-channels with a growing micro-colony (right).

Fig.3 demonstrates three different positions of an initially trapped cell producing a micro-colony. From left to right: growth starts from the bottom of the micro-channel on the surface of the filter (this happens when filter is moistened by media or the micro-channel is filled by media but the initial cell is attached to the filter by adhesion); growth starts at the micro-channel wall where a

cell attaches by forces of adhesion or immunological reaction (micro-channel must be filled by media); cell produces other cells in liquid media, then broth fills the micro-channel separating cells; the initial cell is raised by broth to upper part of micro-channel and starts growth near the border of the liquid media and air.

Fig.4 demonstrates the inner structure and general view of the sampling-detection unit (SDU), the device is used to trap cells by filtration from liquid or air, grow micro-colonies, and treat colonies with chromo- or fluorogenic substrates if needed.

Fig.5 shows the process of filtration using a manifold and attaching nutrient media to the surface of the filter inside the SDU for micro-colony growth. Coloration of micro-colonies is done by agar treated with artificial substrates or dyes can be attached in the same manner.

Fig. 6 shows how micro-colonies in the micro-channels appear under a regular light or fluorescent microscope. It also shows the direction of light through the micro-channels when using fluorescence microscopy.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based on a method and device for trapping cells from liquid or air, allowing for cell growth in a relatively short period of time on solid or liquid nutrient media, treating micro-colonies with chromo- or fluorogenic substrates if desired, and analyzing micro-colonies by microscopy. Micro-channels that contain micro-colonies appear as dark, colored, or fluorescent dots under a regular light or fluorescent microscope depending on the method used. The time of analysis can be reduced, and sensitivity can be enhanced, by using micro-channels of smaller diameter and chromo- or fluorescent substances. Physical factors can also be changed to decrease the time between inoculation and micro-colony detection. For example, heating to

coagulate proteins, increasing light absorbance or light scattering, or adding substances to produce gas bubbles within micro-channels that contain live cells – such as Oxygen (O_2) from Hydrogen Peroxide (H_2O_2) by Catalase – can be employed.

A simple device for trapping cells (Sampling-detection Unit, SDU) in the micro-channels by filtration is shown in Fig.4 and Fig.5. It consists (Fig.4) from a lid (1) with transparent glass or plastic, with one or more very small holes for respiration, a micro-channel plate (2), a filter to trap cells (3), a holder for the filter and micro-channel plate, and a porous support (4) for the filter and micro-channel plate adjusted to the holder (6).

SDU is adjusted to the regular manifold as shown in Fig. 5. The same figure shows different adjustments to the SDU mounted on the manifold for filtration. Thus a funnel for filtration of liquid samples can be adjusted to the filtrating device (left on manifold). A syringe (in the center of the manifold, Fig.5) is used for passing antibody and enzyme conjugates, if identification of micro-colonies is done by enzyme - immunological analysis, or just for passing small samples. The sampling device on the right does not have any additional devices as it is intended for air filtration – trapping bioaerosols (cells and spores) in micro-channels. After filtration is complete, the sampling device is removed from the manifold and placed on a Petri plate with nutrient media(bottom left, Fig. 5) or nutrient media agar cylinder adjusted to lower side of filter (bottom right, Fig. 5) to initiate micro-colony formation.

The procedure for sampling, growing and counting micro-colonies is as following:

- A liquid or air sample containing microorganisms is filtrated through the device. The lid (Fig. 4 - 1) is taken off before filtration, and a special funnel for liquids is adjusted (no funnel is needed for air filtration). During this process, cells, if any, are caught in some of the channels of the micro-channel plate (Fig.4 - 2) on the surface of the filter (Fig.4 -3).
- The support (Fig.4 - 4) adjusted to the holder (Fig.4 - 6) is removed.

- The lid (Fig.4 - 1) is placed on the device to prevent further contamination.

- The holder (Fig.4 - 5) with the micro-channel plate (Fig.4 -2) and the filter (Fig.4 -3) is placed on the surface of an eligible solid nutrient media (Fig.5, bottom – left) or in the container with a liquid nutrient media. The nutrient media is absorbed by the filter and supports the growth of a cylindrical-shaped micro-colony or penetrates through the filter in channels, and supports the growth of suspended microorganisms.

- The device with nutrient media is incubated at an appropriate temperature for the required time for cell growth. In order to reduce the time of analysis by increasing light absorbance or adding fluorescence, the device can be placed in a container with an eligible solution of artificial substrate. Otherwise the substrate can be added to solid nutrient media in advance as it is done in Chromagars.

- The device is placed under a light or fluorescent microscope, and the amount of dark, colored or fluorescent channels is detected and enumerated. This amount corresponds to the number of cells trapped on the surface of the filter. The difference between non-colored micro-colonies, micro-colonies colored by chromogenic substrates or colored dyes, and micro-colonies colored by fluorogenic substrates or fluorescent dyes is shown in Fig. 6.

This invention is capable of detecting a range of microorganisms from a single cell to several hundreds of thousands or even millions, depending on the number of micro-channels in the MCGP. For example, a 25 millimeter square MCGP with micro-channels 10 microns (μm) in diameter (square of plate around 5 cm^2 and 700,000 micro-channels per one cm^2) contains 3.5 millions of micro-channels. Thus, in order to have a reliable enumeration of microorganisms when analyzing the sample, the number of live microbes in a sample should not exceed 350,000 for this plate so that one cell goes to one micro-channel with high level of reliability. If a sample is expected to contain a higher concentration of microbes, it can be diluted. In comparison, a regular

Petri plate limits colony growth from one single cell to only 300 cells/colony as recommended by US Food and Drugs Administration, otherwise colonies will begin to overlap each other and decrease the reliability of enumeration.

This method and device can be used with a broad range of different solid or liquid, natural or artificial media and a large number of different samples.

Example 1.

Detecting and enumerating live bacteria in liquid samples

Detecting and enumerating bacteria in samples is one of the most widely used analyses in medical, environmental and industrial microbiology. Liquids tested include drinking water, beverages, industrial liquids used for manufacturing, natural water, urine, spinal and other body fluids, wash out from surfaces and so on. One of the mostly used nutrient solid media for detection of total viable organisms (mainly bacteria) is Tryptic Soy Agar (TSA). Regular growth of colonies on a Petri plate filled with TSA requires 24 to72 hours at 35°C. Using the proposed invented method; incubation requires only 4 hours. The procedure for the invented technology is as follows:

- The sample (100 milliliters) is filtrated through the SDU, trapping cells in micro-channels containing a colorless MCGP and colorless filter (Polycarbonate, 0.2 microns pores, Osmonics Inc, USA) (Fig. 4, Fig.5). Live cells, if any in the sample, are trapped in some of the micro-channels.
- The SDU is removed from the manifold and a nutrient media (TSA) agar block is attached to the surface of the filter. Nutrient substances from the TSA saturate the filter and penetrate into micro-channels. This process takes around 10-30 seconds.

- The SDU with attached agar block is placed in an incubator for 4 hours at 35°C. Long cylindrical micro-colonies from individual trapped live cells form.
- After incubation, the nutrient media agar block is removed. Another agar block containing Thiazolyl blue tetrazolium salt is adjusted to the filter (a thick filter paper filled by an indicator substance can be used instead). Yellowish molecules of Thiazolyl blue penetrates into the micro-channels. Any micro-channels containing micro-colonies become dark violet in color. The reaction of tetrazolium salt with cells is well-known and based on dehydrogenases of living cells accepting a Hydrogen atom (H+) from the tetrazolium ring for further use in respiration. This reduction then results in a colored substance (Formazan) that collected inside live bacterial cells, mainly in mesosomes. All known bacterial and fungal cells react with tetrazolium salt to reveal this color reaction. Intensely colored long cylindrical micro-colonies are more visible than the same non-colored micro-colonies or colored flat micro-colonies (grown without micro-channels). An agar block (2% in distilled water, 1 cm^3 volume) can be prepared with Thiazolyl blue by adding 3-4 drops of a 3 milligrams per milliliter Thiazolyl blue in phosphate buffer (pH=7.2). Intensively colored cylindrical micro-colonies are visible as colored circles and are easily enumerated in a regular light microscope with a microscopic multiplication from X40 or larger. The concentration (cells per milliliter) of live cells in a sample is calculated by regular known methods.

Thus detection and enumeration of microorganisms in a sample can be completed 6 – 12 times faster than when depending upon cell growth on a regular Petri plate.

Other indicators of live cells can also be used:

- Colorless Fluoresceine diacetate or butirate cleaves by Esterases with the release of highly fluorescent Fluoresceine (green fluorescence = 515 nm). Fluoresceine collects (crystallizes) inside

cells and interrupts biochemical pathways, which cause cell death. Thus, Fluoresceine diacetate and other Fluoresceine derivatives can be used after micro-colonies are formed.

- Colorless 4-Methylumbelliferyl acetate, -butyrate, -propionate, or -phosphate cleaves by Esterases, Lipases or Phosphatases with the release of 4-Methylumbelliferone, a highly fluorescent substance (blue fluorescence = 450 nm). 4-Methylumbelliferone is secreted from cells and concentrates in extracellular spaces, filling the remaining volume of the micro-channel. Therefore, these fluorogenic substrates can be used during micro-colony growth. Simultaneously incubating a sample and allowing cell growth while treating cells with 4-Methylumbelliferone, and thus allowing an extracellular buildup of fluorescent signal, can significantly reduce the time required for analysis as very small micro-colonies (10-20 cells) can be detected.

- Other than Thiazolyl blue, Tetrazolium salts such as Tetrazolium iodo (INT), Nitrotetrazolium blue (NBT), and BT-tetrazolium that reveal differently colored Formazans can also be employed. Nevertheless, these salts are not as universal as Thiazolyl blue and, therefore, can be used for certain samples.

- Chromogenic substrates such as 5-Bromo-4-chloro-3-indoxyl butyrate, - palmitate, -phosphate (blue precipitates inside cell) or 6-Chloro-3-indoxyl butyrate, -palmitate (red color precipitates) for Esterases, Lipases, or Phosphatases, as well as other chromogenic substrates can be used for coloration of formed micro-colonies and as additives to nutrient media. Chromogenic substrates that are dissolved in nutrient media before application for cell growth are referred to as "Chromagars." However, Chromagars are created for only a few microorganisms: CHROMagar™ Candida, CROMagar™ O157, CHROMagar™ Salmonella, CHROMagar™ Staph aureus and CHROMagar™ Orientation for urinary tract pathogenic microorganisms.

- Dyes, such as Dansylchloride (DNS-chloride) or Fluorescamine, are capable of increasing fluorescence hundreds- or even thousands-fold after attaching to biomolecules such as NH- groups of proteins. These compounds are also useful for marking micro-colonies for enumeration.
- Some substances are known to change the color of colonies to a dark or even black color, making micro-colonies more visible on a Petri plate or under a microscope in micro-channels. For example, iron sulfide in SPS Agar is known to color Clostridia, and XLT4 Agar Base colors Salmonella. Likewise, potassium telluride in VJ Agar colors Staphylococcus aureus colonies.

Growth of more than 4 hours is often enough to produce long micro-colonies, and detect and enumerate a large number of non-colored micro-colonies by their enhanced light absorbance or light scattering. Also, light absorbance and light scattering of micro-colonies in micro-channels can be increased by high temperature (proteins coagulation) or addition of reagents that produce bubbles in the presence of live cells (i.e., Catalase – Hydrogen Peroxide) and are known to have good refractivity and therefore light scattering.

Many different kinds of samples, a variety of microorganisms, hundreds of nutrient media, and a multitude of indicator substances opens a huge opportunity for the implementation of this invented technology in the different areas of microbiology.

Example 2.

Identification of micro-colony of E.coli O:157 by enzyme immunoassay in micro-channels

Micro-colonies can be identified in the micro-channels using enzyme immunological analysis (EIA). Cells are trapped in the micro-channels by filtering a sample as described in Example 1. The use of EIA for the identification of micro-colonies is based on the immunological

reaction between antigens of the cells (micro-colony) and enzyme-antibody conjugates. The conjugate is passed through the micro-channels in order to perform antibody – antigen reactions. The syringe shown in Fig.5 is suitable for this because it allows a small volume of conjugate to be slowly pressed through the micro-channels. The micro-colonies in this case must be very small, 8-32 cells, as larger micro-colonies can clog the micro-channel. After the conjugate attaches to E. coli O:157 antigens, a block of pure agar filled by Tetramethylbenzidine (a substrate for Horseradish Peroxidase – an enzyme of the conjugate) is attached to the filter. Tertamethylbenzidine is cleaved by the Horseradish Peroxidase with creation of a blue-colored dye that collected in micro-channel with E.coli O:157. The number of E.coli O:15 present in the sample corresponds to the number of blue micro-channels. This example is based on a well known color reaction, but fluorescent reactions are also available. Thus a conjugate consisting from antibody and ß-D-Galactosidase gives a fluorescent 4-Methylumbelliferone in reaction with 4-Methylumbelliferyl-ß-D-Galactose. Conjugates consisting from an antibody and Phosphatase produce 4-Methylumbelliferone in reaction with 4-Methylumbellyferyl phosphate, disodium salt.

Example 3.

Sampling, detection and enumeration of bioaerosols.

MCA technology can also be used for the rapid analysis of bioaerosols. Air is filtrated through the SDU, which is adjusted to the manifold (Fig. 6, right holder). The manifold is connected to an air pump (AirCheck HV30, QuickTake 30 or another, SKC Inc., USA). A rotameter for measuring the air volume is installed between the manifold and the pump. A required volume of air is passed through the SDU, and microorganisms present in the air sample

are trapped in the micro-channels. Bacilli and Fungi spores are considered the main microorganisms in bioaerosols. Thus, two nutrient media need to be used, TSA for Bacilli and SDA for Fungi spores. This also requires using two SDU. Dormant spores of Bacilli usually germinate within 0.5-1.5 hours after contact with a nutrient media. This time needs to be added to the regular time of Bacilli incubation trapped in micro-channels (4 hours) in order to form a cylindrical micro-colony from the spore. Germination of Fungi spore requires about 2-8 hours, followed by incubation in the SDU of about 12 hours. After micro-colonies from spores appear in the micro-channels, procedures described in Example 1 (detection of the total number of viable microorganisms) or Example 2 (identification of micro-colonies) or other procedures developed for micro-channel analysis take place. Another version of sampling air microorganisms is by first sampling in liquid (sodium chloride solution, buffer, liquid nutrient media) with help of well-known liquid samplers (e.g., AGI-30, SKC BioSampler) and then filtrated through the SDU. Currently used methods for bioaerosol detection are based on "landing" particles on the surface of agar media (Impactor BioStage, SKC Inc.) or inoculating liquid samples with microorganisms sampled beforehand. Both methods need a long growth period for the microorganisms in order to form well visible colonies: 24-72 hours for bacteria and 72-120 hours for fungi.